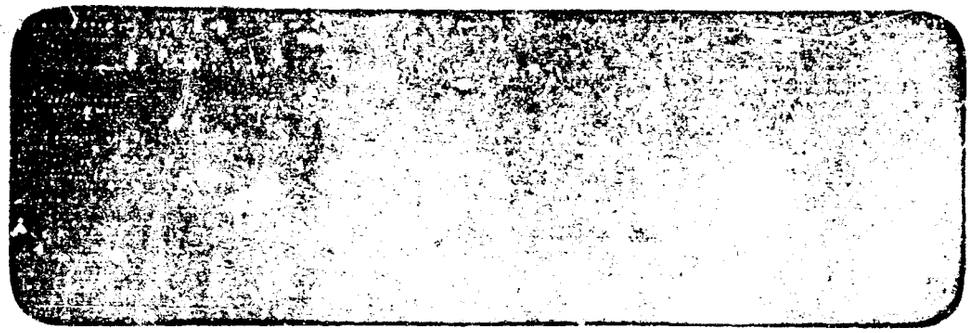

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Sporicidal Activity of Peracetic Acid and β-Propiolactone at Subzero Temperatures

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A method was developed to evaluate and measure the sporicidal activity of peracetic acid (PAA) and β-propiolactone (BPL) at subzero temperatures as low as −40 C. *Bacillus subtilis* var. *niger* spores were used as the test organism. Both PAA and BPL were sporicidal at low temperatures, with PAA the more active. The temperature coefficients of the two chemicals are generally low over a range of 20 to −20 C, but increase significantly at temperatures below this. Results showed an initial lag in the PAA death rates that was directly dependent on the temperature. BPL did not show this lag time.

The literature on chemical disinfection is voluminous, but information on disinfection at very low temperatures is limited. Most of the data reported on disinfection below ambient temperatures have been on chlorine and chlorine derivatives in the range of 1 to 4 C. Early studies on chlorine and chloramine were made by Heathman et al. (10) and Butterfield (2) at 2 to 5 C on vegetative cells. Brazis et al. (1) investigated the effects of free available chlorine on *Bacillus subtilis* var. *niger* (*B. globigii*) and *B. anthracis* spores at 4 C. Fetner and Ingols (4) tested the effect of ozone and chlorine on *Escherichia coli* at 1 C. Gorzhkovskaya (6) reported the use of calcium hypochlorite, under practical conditions, with common salt added to prevent freezing. This solution was effective over a short period of time but not after the disinfectant began freezing at −12 C. Shura-Bura (17) reported good sporicidal effect at −12 C with active chlorine produced by electrolyzed solutions of sodium chloride.

These reports indicate that there is a need for testing new chemicals and developing new methods for testing those chemicals that show promise as low-temperature disinfectants. Recent studies in this laboratory showed peracetic acid (PAA) and, to a lesser extent, β-propiolactone (BPL) to be very active against *Serratia marcescens* at 4 C.

Greenspan and MacKellar (9) and Hutchings and Xezones (Bacteriol. Proc., 1949, p. 50) reported PAA to be a bactericide, fungicide, and sporicide. In 1958, BPL was reported as a vapor-phase disinfectant by Hoffman and Warshowsky (11). Since that time, the bactericidal effect of PAA and BPL has been studied by numerous investigators; PAA and BPL have been shown to possess germicidal properties when employed in a gaseous or liquid state (3-5, 9, 11, 15, 16). On the basis of preliminary tests on their sporicidal effect, PAA and BPL were chosen as candidate chemicals for this study. This paper is concerned with developing and evaluating a method to measure the sporicidal activity of PAA and BPL at temperatures in the range of 20 to −40 C. *B. subtilis* var. *niger* spores were used as the test organism because of their resistance to chemical action, ease of standardization, and the abundant data on their performance at higher temperatures.

Materials and Methods

Apparatus. The apparatus (manufactured by Miller and Murphy, Inc., Chicago, Ill.) used for this study contained a chamber in which the temperature could be controlled between 5 and −30 C. To obtain lower temperatures, the apparatus was modified by inserting a double-walled insulated rectangular steel box lined with an evaporator coil connected to a ¾-hp condensing unit outside the refrigerator (Fig. 1). The condenser allowed the temperature to be lowered and maintained as low as −45 C. The steel box, which was filled with a mixture of ethylene glycol and water, had a perforated metal shelf to support the test bottles. A canister of sterile pipettes was placed in the chamber and allowed to equilibrate to the various temperatures to minimize temperature change during sampling. All samples were withdrawn with a bulb pipette as a safety precaution against chemical vapors.

Ethylene glycol. To prevent freezing at low temperatures, ethylene glycol, reagent grade, was added to spore suspensions and test chemical solutions in the percentages shown in Table 1. Ethylene glycol was tested for sporicidal activity and was found to have no effect on germination and growth of *B. subtilis* spores at the test concentrations and temperatures. Although

ordinary glycols are among the least toxic of many compounds used to protect against freeze damage to living cells, ethylene glycol has been shown to be bactericidal against airborne vegetative cells. However, in low aqueous concentration it has only minor bactericidal activity. 15, 16).

Preparation of test solutions. Solutions of PAA and BPL were prepared from concentrated commercial products, and the exact concentrations were determined by the specific iodometric procedures described by Greenspan and MacKellar for PAA (8) and Tyler and Beesing for BPL (18). The test solutions were prepared in volumes that were ¾ as strong as the desired concentrations, so that when the solution was added to the spore suspension in a ratio of 4 to 1 the test mixture would be of the desired concentration. To prevent freezing, both the test solution and the spore suspension had the same concentration of ethylene glycol prior to mixing for subzero tests.

Spore suspension. The *B. subtilis* spore suspension was prepared from a concentrated preparation that was washed and diluted to a final test concentration of about 10^8 organisms per milliliter. Spore stock suspensions were stored in the refrigerator for several weeks in 53% ethylene glycol without any discernible adverse effects.

Neutralizer. Sodium thiosulfate (1 M) was used as the chemical neutralizer for PAA and BPL. The proper concentrations of thiosulfate were prepared so that there was only a small excess remaining after the PAA and BPL were neutralized prior to plating; 5-ml volumes of the proper concentrations of thiosulfate were used to neutralize 5-ml samples of the test mixture, bringing the total volume of the neutralized sample in the dilution tube to 10 ml.

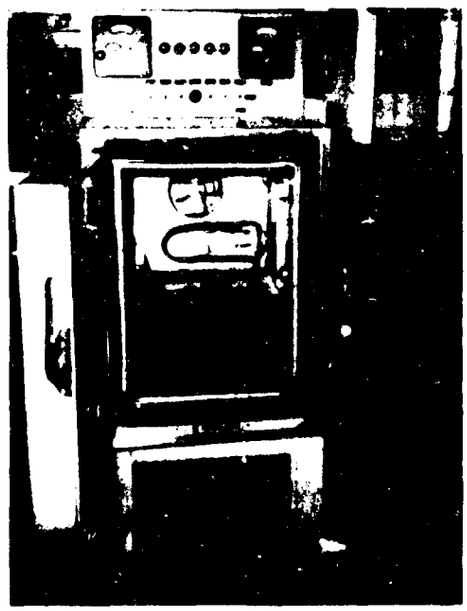

FIG. 1. *Low-temperature apparatus*

TABLE 1. *Ethylene glycol concentrations used at various temperatures*

Temp	Ethylene glycol
C	%
10	0
0	5
−10	25
−20	36
−30	45
−40	53

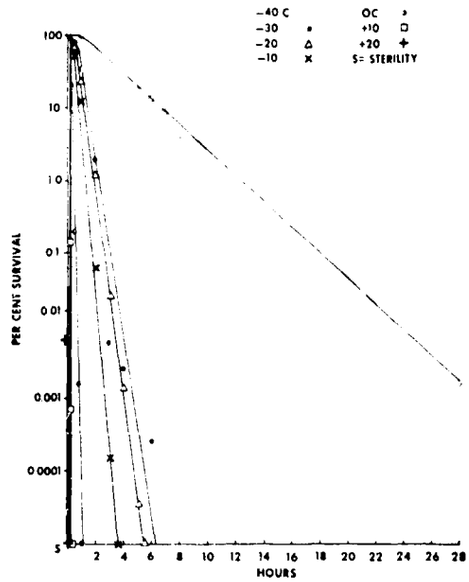

FIG. 2. *Survival of Bacillus subtilis spores exposed to 0.3% peracetic acid at indicated temperatures.*

Procedure. The PAA or BPL solutions and spore suspensions in separate 200-ml screw-cap bottles were placed in the ethylene glycol bath and equilibrated to the proper test temperature. The control spores and distilled water (no decontaminant), with the same concentration of ethylene glycol used in the test solution, were equilibrated at the same time. After the equilibration period, the test chemical solution was poured directly into the spore suspension, capped, mixed thoroughly, and sampled for viable microorganisms. The *p*H and concentration of the PAA or BPL solutions were determined prior to mixing with the pores, just after mixing, and again at the end of the test period. No appreciable change in *p*H or drop in disinfectant concentration was noted.

Samples (5 ml) of the test solution with spores were removed as desired and placed into neutralizer, serially diluted, and plated. At the start of the test, a

5-ml sample was withdrawn from the spore control, placed in thiosulfate, and plated in the same manner as the test mixture. The pour-plate method was employed, with Tryptose Agar (Difco). Plates were incubated at 37 C and counted at 48 hr.

In these experiments, 0.3% PAA was evaluated at intervals of 10 C between 20 and −40 C; 3% PAA was evaluated in the same manner at temperatures between 0 and −40 C. BPL solutions of 4, 10, and 20% were prepared and evaluated at intervals of 10 C between 20 and −20 C. In all, 50 samples of PAA and 18 of BPL were tested.

The sporicidal activity of these chemicals was measured by determining the rate at which viable spore counts dropped in various concentrations of the chemical at different temperatures. The death rates are expressed in graphic form as log per cent survival versus time. The controls showed no decrease in viable spore count in the various ethylene glycol solutions at the tested temperatures and exposure times. For the purpose of this paper, a 7-log reduction in the spore population was considered sterility.

RESULTS

With the addition of ethylene glycol to aqueous solutions of PAA and BPL, it is evident that *B. subtilis* spores can be sterilized at subzero temperatures (Fig. 2-5). All death rates are straight-line functions, although there is an initial lag in the PAA curves that does not occur with BPL. PAA exhibits excellent disinfectant properties over the temperature range of 0 to −30 C in fairly low concentration, but requires a considerably longer time at −40 C to sterilize.

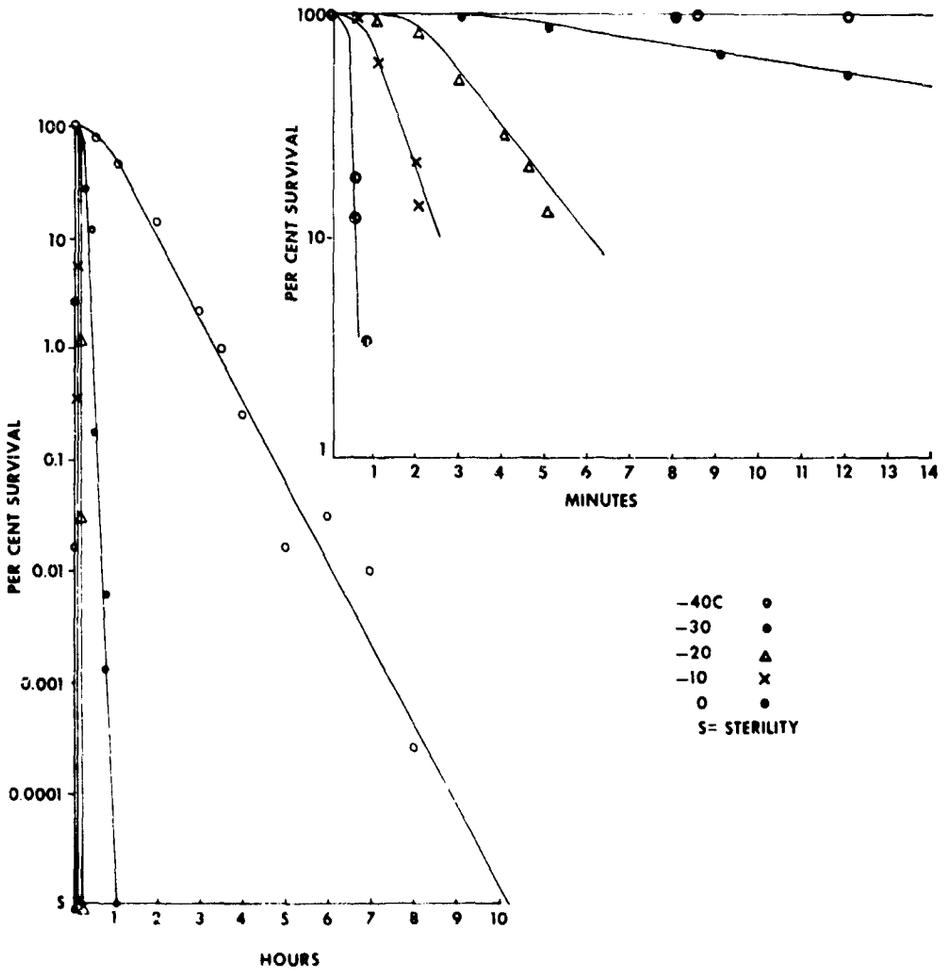

FIG. 3. *Survival of Bacillus subtilis spores exposed to 3% peracetic acid at indicated temperatures*

BPL, on the other hand, shows sporicidal activity at subzero temperatures, but requires a much longer time to sterilize than does PAA. Table 2 shows that 0.3 and 3% PAA solutions are 20 times and 574 times, respectively, more active than 10% BPL at −20 C.

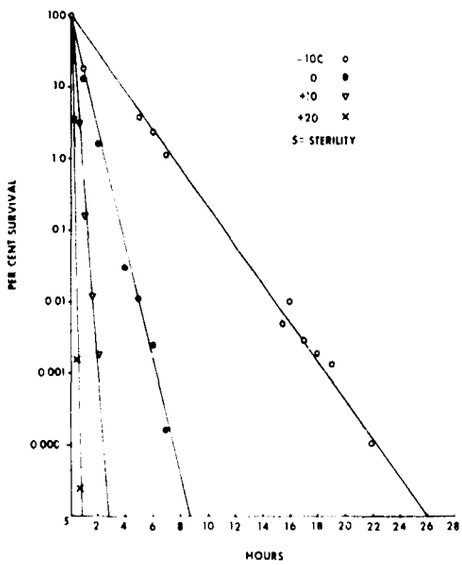

FIG. 4. *Survival of Bacillus subtilis spores exposed to 4% BPL at indicated temperatures.*

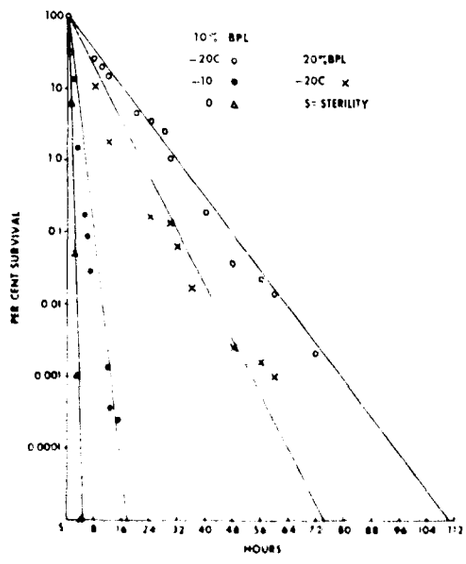

FIG. 5. *Survival of Bacillus subtilis spores exposed to 10 and 20% BPL at indicated temperatures.*

TABLE 2. *Comparative activity of peracetic acid (PAA) and β-propiolactone (BPL) based on the ratio of the time required for sterility*

%PAA/%BPL	Activity ratio[a]	
	−10C	−20 C
0.3/4.0	7.3	—
0.3/10.0	4.6	20
0.3/20.0	—	12.4
3.0/4.0	312	—
3.0/10.0	168	574
3.0/20.0	—	355

[a] For comparison, the ratio of activity of 3% PAA to 0.3% PAA was: −40 C, 5; −30 C, 6.6; −20 C, 28.6; −10 C, 42.6; 0 C, 40.6.

TABLE 3. *Temperature coefficients (Q_{10}) for various concentrations of PAA and BPL*[a]

Temp differential	Q_{10}			
	0.3% PAA	3.0% PAA	4% BPL	10% BPL
C				
−40 to −30	6.6	10.0	—	—
−30 to −20	1.2	5.2	—	—
−20 to −10	1.5	2.0	—	6.3
−10 to 0	3.5	3.3	5.0	4.0
0 to 10	5.0	—	3.2	—
10 to 20	2.4	—	3.5	—

[a] Ratio of times required for sterility at temperature differentials of 10 C.

Temperature coefficients, Q_{10} values, were calculated by dividing the time required to sterilize the spore cultures at a temperature t by the time required for a similar culture at t plus 10 C (Table 3). The Q_{10} for 3% PAA over the 0 to −20 C temperature range is about 2.7, but increases over the −20 to −40 C range. The 0.3% PAA shows variable Q_{10} values and a considerable increase between −30 and −40 C, which further demonstrates the effect of temperature on the rate of disinfection. The same general trend of higher Q_{10} values at lower temperatures was exhibited by 10% BPL.

In the curves for PAA, there is a period of no sporicidal activity at the beginning of contact. The length of this lag period is proportional to the temperature; that is, the lower the temperature, the longer the lag. At the end of the lag period, there is an increase in the death rate up to a point, and it then continues in an orderly manner until sterility. No such lag period is seen in the BPL curves.

When the log of the lag time versus the tempera-

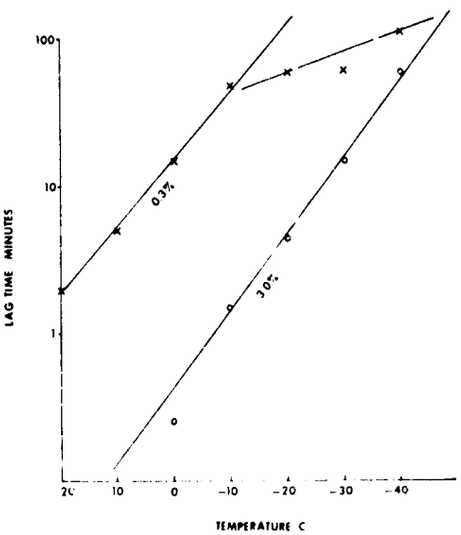

FIG. 6. *Temperature-lag time relationship for peracetic acid.*

ture is plotted (Fig. 6) for 3 and 0.3% PAA, two straight and almost parallel lines are obtained. At −20 C, the 0.3% PAA curve deviates and bends toward the curve of the higher concentration, which appears as a second lag phase.

DISCUSSION

In studying low-temperature disinfection, certain conditions must be met. First, the test solution must be kept fluid at subzero temperatures by adding an antifreeze that is compatible with the chemical and nontoxic to the bacterial agent Second, there must be adequate temperature control at the subzero temperatures tested, with rapid transfer of samples to a neutralizer to halt the chemical activity immediately and to allow accurate measurement of the death rate.

Both of these conditions have been satisfactorily met in this study, with the possible exception of the inhibitory effect of PAA. PAA showed a partial inhibitory effect with all concentrations used; this was expressed in the lower plating dilutions, for example, 1:10 and 1:100. The degree of inhibition increased with an increase in PAA concentration, and this effect could be demonstrated by serially diluting and pour-plating the neutralized PAA spore mixture.

Attempts to clarify all the factors producing inhibition were unsuccessful. There was evidence of an interaction between PAA and ethylene glycol, resulting in the formation of aldehydes; however, this could not account for the observed inhibition. When PAA was allowed to decompose in the presence and absence of ethylene glycol for 7 months at ambient temperatures, the inhibitory effect was still present in both preparations, including the one that did not contain ethylene glycol. The activity was not greater than that observed at the start of the test. PAA concentration had dropped from 3 to 0.08%, but this lower concentration still possessed sporicidal activity; thiosulfate was required to neutralize residual PAA. However, the inhibitory effect and the killing effect can be measured separately, and the sporicidal activity continues independently of the inhibitory activity.

The high concentrations of BPL when neutralized did not exhibit inhibitory properties.

Both PAA and BPL are shown to be sporicidal at low temperatures, but PAA is by far the more active of the two. The temperature coefficients of the two chemicals are generally low over a range of 20 to −20 C; they increase significantly at temperatures below this. It is thus evident that it would be dangerous to predict microbial death rates at lower temperatures merely by extrapolation from a temperature coefficient obtained at higher temperatures.

Of considerable interest is the initial lag shown in the PAA death rates. The lag time is directly dependent on the temperature; the lower the temperature, the longer the lag time.

In studying the problem of the lag phase of log per cent survival with time, a point is selected that approximately separates this phase. Then log survival, beyond this point, has a close linear relationship with time.

A line was fitted to cases beyond the selected point and extended backward to zero-time; deviations from the line of these cases were then measured. Since these deviations ranged in magnitude from 2.5 to 5 times the standard error, statistically they do not belong on the line. This gives sufficient proof that there is a different slope in the early part of the trend.

Whether such a relationship is characteristic of oxidizing agents in general is not known; however, Brazis et al. (1) and Levine (12) reported lag periods in their death rate studies with chlorine. Their studies were not carried out at a sufficient number of temperatures to determine whether there was a direct relationship between lag time and temperature. Studies with this chemical, however, are currently under way in this laboratory, and it will be determined whether there is a relationship between time lag and temperature.

ACKNOWLEDGMENT

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